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Review

## CDP-Choline Production from CMP and Choline by Yeasts

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### INTRODUCTION

CDP-choline is known as an important intermediate of lecithin biosynthesis and as an important drug for nervous diseases of the brain.

In 1952, Kornberg and Pricer<sup>1)</sup> reported in a preliminary communication that phosphoryl choline labeled with <sup>32</sup>P and <sup>14</sup>C was converted by liver enzyme to a lipid which was not further characterized. The work of Kennedy and Weiss<sup>2-5)</sup> has shown that CDP-choline and CDP-ethanolamine are naturally occurring coenzyme forms of P-choline and P-ethanolamine, and that these nucleotides are precursors of lecithin and phosphatidyl ethanolamine, respectively. The synthesis of lecithin has been shown to take place according to the following equations:

- 1)  $\text{CTP} + \text{P-choline} \longrightarrow \text{CDP-choline} + \text{Pyrophosphate}$
- 2)  $\text{CDP-choline} + \text{D-}\alpha\text{-}\beta\text{-diglyceride} \longrightarrow \text{Lecithin} + \text{CMP}$

In similar reactions, phosphatidyl ethanolamine is synthesized from P-ethanolamine *via* cytidine diphosphate ethanolamine. Since the reaction shown in equation(1) is essentially a transfer of P-choline to the cytidyl portion of CTP, the enzyme catalyzing the reaction has been named phosphorylcholine-cytidyl transferase.<sup>4)</sup> The enzyme catalyzing reaction (2) has been called phosphorylcholine-glyceride transferase.

Borkenhagen and Kennedy<sup>6)</sup> have reported the preparation and some properties of phosphorylcholine-cytidyl transferase of guinea pig liver, and suggested that a separate enzyme, phosphorylethanolamine cytidyl transferase, catalyzed the synthesis of cytidine diphosphate ethanolamine from CTP and phosphorylethanolamine by an analogous reaction.

CDP-choline, cold or labeled with <sup>14</sup>C or <sup>32</sup>P, which was employed for the investigation of enzymatic incorporation of choline or inorganic phosphate into the phosphatides, has been prepared mainly by chemical means.<sup>7-9)</sup>

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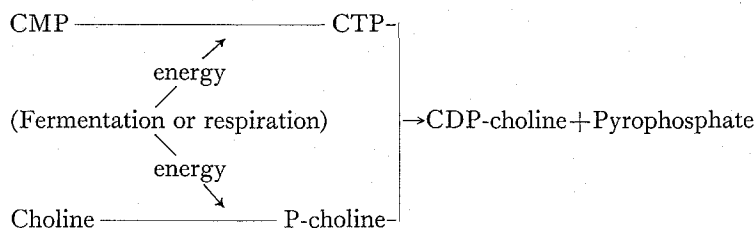
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The following abbreviations have been used:

CDP-choline, cytidine diphosphate choline; CTP, cytidine 5'-triphosphate; P-choline, phosphoryl choline; CMP, cytidine 5'-monophosphate; FBP, fructose 1,6-*bis* phosphate; CDP, cytidine 5'-diphosphate.

Recently, Tochikura *et al* reported the fermentative production of CDP-choline and CDP-ethanolamine from CMP and P-choline<sup>10-12)</sup> or CMP and P-ethanolamine<sup>13,14)</sup> by use of dried cell preparation of yeasts.

This paper describes the fermentative production of CDP-choline from CMP and choline by use of the phosphorylation energy derived from glucose dissolution by dried yeast cells in the presence of high levels of inorganic phosphate. The mechanism of the fermentative production of CDP-choline is postulated in the following scheme. The reaction mechanism of energy supply is based on the Harden-Young effect<sup>15)</sup> of glucose metabolism and on the method which has been employed for fermentative production of sugar nucleotides.<sup>16-18)</sup>



## MATERIALS AND METHODS

**Chemicals** CMP- $\text{Na}_2$  was kindly supplied by Kyowa Hakko Kogyo Co. Ltd., Tokyo. All other chemicals used were commercial products.

**Microorganisms and cultivation** *Hansenula jadinii* IFO 0987 was mainly used for the production of CDP-choline. A screening test for CDP-choline production from choline and CMP was performed with other strains of yeast which were capable of phosphorylation of choline. All the yeasts were grown on the medium containing 5% glucose, 0.3% peptone, 0.2% yeast extract, 0.2%  $\text{KH}_2\text{PO}_4$ , 0.3%  $(\text{NH}_4)_2\text{HPO}_4$ , and 0.1%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .

The cultivation was carried out at 28°C for 24 to 48 hr on a reciprocal shaker with 2 liter shaking flasks containing 500 ml of the above medium. The cells harvested by centrifugation were washed three times with deionized water and air-dried at room temperature for 24 to 48 hr with an electric fan, followed by desiccation over night under reduced pressure over  $\text{P}_2\text{O}_5$ . The dried cells were kept at -20°C until use.

**Analysis** The amount of cytidine nucleotides was determined by measuring their optical density at 280 nm by use of the molar absorptivity ( $13 \times 10^3$ ) for cytidine after extracting these compounds from the spots on paper chromatograms with 0.01 N HCl. Paper chromatography was carried out on Toyo filter paper No. 53 using a solvent system which was composed of 95% ethanol and 1 M ammonium acetate (2 : 1, pH 7.5).<sup>19)</sup> The solvent systems containing *iso* butyric acid and 0.5 N ammonia (10 : 6),<sup>20)</sup> *iso* propanol: conc. ammonia:  $\text{H}_2\text{O}$  (7 : 1 : 2),<sup>8)</sup> phenol (50 g) + *n*-butanol (50 ml) + 80% formic acid (3 ml) +  $\text{H}_2\text{O}$  (10 ml),<sup>21)</sup> were used for the determination of aminoethanol. Choline was detected as a yellow spot on the paper chromatogram or thin layer chromatogram of silica gel G by spraying with alkaline permanganate solution.<sup>22)</sup> Solvent system used was methanol: conc. HCl (95 : 5).

P-choline or P-ethanolamine was separated by paper chromatography using a solvent

system of 95% ethanol: 1 M ammonium acetate (2 : 1) and detected by treatment with Hanes-Isherwood reagent.<sup>23)</sup>

The area corresponding to P-choline or P-ethanolamine of a simultaneously developed untreated paper was cut out and transferred to a test tube containing 3 ml of Tris-HCl buffer (pH 8.0). An aliquot (50  $\mu$ g protein) of alkaline phosphatase from *E. coli*<sup>24)</sup> was added to the tube which was kept at 30°C for 3 hr. Inorganic phosphate liberated was assayed by the method of Fiske-SubbaRow.<sup>25)</sup> Choline kinase activity under fermentative conditions was shown by the production of P-choline or P-ethanolamine in the standard reaction system of CDP-choline fermentation from which CMP was omitted. Glucose was determined by the method of Somogyi<sup>26)</sup> and FBP was assayed by the method of Roe.<sup>27)</sup>

**Standard reaction system** The standard reaction system for the screening test contained 20 mM CMP, 50 mM choline chloride (or ethanolamine in the case of choline kinase assay), 200 mM phosphate buffer (pH 7.4), 400 mM glucose, 12 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and 100 mg per ml of dried cells in a total volume of 2.0 ml. Reaction was carried out at 28°C with continuous shaking and was terminated by immersing the tubes in boiling water for 3 min before cooling. The cell debris was removed by centrifugation and the supernatant solution was analysed.

**Isolation of the reaction products** CDP-choline formed from CMP and choline was separated by column chromatography with Dowex-1, X-2 (formate form) by the modified method of Tochikura *et al.*<sup>10)</sup>

After centrifugation, the supernatant of the reaction mixture was adjusted to pH 2.0 by addition of conc. HCl, and was again centrifuged at 6,000 rpm for 30 min at 0°C. Activated charcoal, onto which cytidine nucleotides are quantitatively absorbed, was added to the supernatant. The amount of activated charcoal added depended on the absorbance of the supernatant, 100 mg being added for an absorbance of 200 at 280 nm. The mixture was stirred at 5°C overnight. The charcoal was collected by filtration and washed with acidic cold water. Nucleotides were extracted from the charcoal by mixing with 50% alcohol containing 2% of ammonia for several hr. The filtrate was condensed to a small volume under decreased pressure at below 50°C, and the condensate was applied to a column of Dowex-1, X-2 (formate form 1.8  $\times$  48 cm).

CDP-choline was separated from other cytidine nucleotides by elution with  $2.5 \times 10^{-3}$  M formic acid. Fractions containing CDP-choline were combined and were absorbed again on an appropriate amount of activated charcoal, decided from the absorbance at 280 nm. CDP-choline was eluted again from charcoal by the above procedure. The eluate was condensed and the condensate was chromatographed through a column of Dowex-50 (Na form).

The fractions corresponding to the CDP-choline were combined and condensed to a small volume. The condensate was again treated with a small amount of activated charcoal to decolorize it. The condensate was diluted 4 to 5 times with absolute alcohol, and the mixture was kept in a cold room for a few days.

White plate-like crystals appeared which were collected by filtration. Recrystallization was performed by the method stated above.

## RESULTS

*Distribution of CDP-choline forming activity in yeasts* The distribution of CDP-

choline forming activity in 38 strains of yeasts was investigated using the standard reaction system. The results are summarized in Table I (column A). It is of interest that relatively

Table I. Distribution of Choline kinase and CDP-choline Forming Activity in Yeasts.

	CDP+CTP	Nucleotides found in 6 hr incubation (mM)		choline kinase*
		CDP-choline (A)	(B)	
<i>Brettanomyces clausenii</i> (IFO 0627)	1.64	1.99	4.84	0.5
<i>Candida krusei</i> (IFO 0013)	7.22	2.31	3.66	2
<i>Candida parapsilosis</i> (IFO 0708)	13.00	4.05	5.29	—
<i>Candida pseudotropicalis</i> (IFO 0617)	2.09	1.95	2.43	1
<i>Candida rugosa</i> (IFO 0591)	trace	trace	—	0
<i>Candida tropicalis</i> (IFO 0006)	13.40	1.45	4.45	—
<i>Candida utilis</i> (IFO 0396)	2.40	5.10	5.02	13
<i>Candida utilis</i> (IFO 0639)	2.99	6.39	6.39	15
<i>Candida utilis</i> (IFO 1086)	3.93	4.63	5.50	160
<i>Debaryomyces globosus</i> (IFO 0016)	7.71	0.93	2.60	—
<i>Debaryomyces hansenii</i> (IFO 0023)	14.10	2.95	3.55	—
<i>Debaryomyces subglobosus</i> (IFO 0794)	10.70	4.92	4.50	—
<i>Debaryomyces vini</i> (Y.U.)	18.45	1.93	4.00	0.5
<i>Endomyces hordei</i> (IFO 0104)	1.44	0.58	1.80	0.5
<i>Eremusculus fertilis</i> (IFO 0691)	13.20	2.40	2.46	1
<i>Hanseniaspora valbyensis</i> (IFO 0115)	17.40	3.35	2.02	—
<i>Hansenula anomala</i> (IFO 0149)	2.08	0.81	3.09	—
<i>Hansenula anomala</i> (IFO 0569)	2.03	1.35	1.35	—
<i>Hansenula beijerinckii</i> (IFO 0981)	8.70	5.09	5.95	1
<i>Hansenula bimundalis</i> (IFO 1366)	10.02	2.30	3.00	1
<i>Hansenula jadinii</i> (IFO 0987)	6.75	9.15	9.00	100
<i>Hansenula matritensis</i> (IFO 0954)	17.00	1.83	2.90	—
<i>Hansenula miso</i> (IFO 0146)	1.80	6.50	6.95	—
<i>Hansenula mrakii</i> (IFO 0985)	15.21	2.82	4.05	—
<i>Hansenula octosporus</i> (IFO 0145)	6.60	2.56	4.82	14
<i>Hansenula petterssonii</i> (IFO 1372)	14.78	0.58	1.52	6
<i>Hansenula saturunus</i> (IFO 0117)	19.70	0.87	1.52	10
<i>Hansenula silvicola</i> (IFO 0807)	0.68	0	1.16	—
<i>Hansenula wingei</i> (IFO 0976)	2.96	1.26	2.59	8
<i>Lipomyces lipoferus</i> (IFO 0673)	trace	trace	trace	—
<i>Pichia polymorpha</i> (IFO 0195)	2.80	3.00	3.64	17
<i>Rhodotorula glutinis</i> (IFO 0388)	0	0	—	—
<i>Rhodotorula rubra</i> (IFO 0889)	4.30	1.54	1.93	0.5
<i>Saccharomyces carlsbergensis</i> (IFO 0641)	14.40	4.75	4.65	1.5
<i>Saccharomyces cerevisiae</i> (IFO 0021)	4.81	5.20	6.50	—
<i>Saccharomyces rouxii</i> (IFO 0320)	14.40	4.45	5.80	1
<i>Saccharomyces rouxii</i> (IAM 4369)	13.15	6.38	6.93	1
<i>Torulopsis candida</i> (IFO 0768)	18.20	1.83	—	0

\* Relative activity of choline kinase was determined by phosphorylation of ethanolamine under the fermentation conditions. One hundred per cent activity corresponds to a phosphorylation of ethanolamine of 3.32  $\mu$ moles/ml/hr.

(A), no supplementation of *T. candida*; (B), *T. candida* supplemented (100 mg/ml).

high activity of CDP-choline formation was found widely among *Candida* species, *Hansenula* species, and *Saccharomyces* species. With *Candida utilis* IFO 0396, IFO 0639, IFO 1086, *Hansenula beijerinckii* IFO 0981, *H. jadinii* IFO 0987, *H. miso* IFO 0146, *Saccharomyces carlsbergensis* IFO 0641, *S. cerevisiae* IFO 0021 and *S. rouxii* IFO 0320 and IAM 4369, 4 to 9 mM of CDP-choline was formed during 6 hr incubation.

These strains are known to be capable of forming CDP-choline from P-choline and CMP, but produced lesser amounts of CDP-choline from choline and CMP.

Since *T. candida* showed high activity of CDP-choline formation from P-choline and CMP<sup>11)</sup> but little activity was observed from choline and CMP, the effect of addition of *T. candida* cells into the reaction tube of other strains as a source of CMP kinase and phosphorylcholine-cytidyl transferase was tested. As shown in Table I (column B), in some strains such as *Brettanomyces clausenii*, *C. tropicalis*, *D. vini*, and *H. anomala*, the amount of CDP-choline formed was enhanced 1.2 to 1.5 times.

Choline kinase activity was examined and expressed in Table I by phosphorylation of ethanolamine during fermentation without addition of CMP. High activity of choline kinase was observed in *C. utilis* IFO 1086 and *H. jadinii* IFO 0987. The others showed relatively low activity of ethanolamine phosphorylation. The results presented here suggest that none of the strains had an excess activity of choline kinase.

Using the dried cell preparation of *H. jadinii* IFO 0987, the optimum conditions for the fermentative production of CDP-choline from CMP and choline were investigated.

#### Effect of various factors on CDP-choline formation

1) *Effect of pH*: As shown in Fig. 1, CDP-choline production was strictly dependent

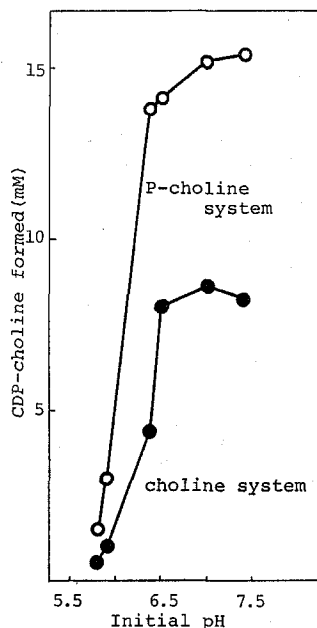


Fig. 1. Effect of initial pH of the reaction mixture on CDP-choline formation. The reaction mixture (mM): CMP, 20; glucose, 400; potassium phosphate (initial pH was indicated above), 200;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 10; choline (in choline system), 50; P-choline (in P-choline system), 50. Cells 100 mg per ml. Total volume 2.0 ml. Shaken at 28°C for 8 hr.

on the initial pH of the reaction mixture. CDP-choline formation was examined at various pHs in phosphate buffer of pH 6.0, 6.5, 7.0, 7.5, 8.0, and 8.9. The initial pH, measured 30 min after the reaction started, of the reaction mixtures was 5.8, 5.9, 6.4, 6.5, 7.1, and 7.4, respectively.

The optimum pH of the initial reaction mixture was between 6.5 and 7.4, the maximum yield of CDP-choline being about 8 mM. The amount of CDP-choline formed from choline and CMP at the initial pH of 6.4 was about 50% of that at pH 6.5, and little product was found at pH 5.9 and 5.8. The production from P-choline at the initial pH of 6.4 was not so decreased.

Chemical changes in the reaction mixture were examined. As shown in Fig. 2, more than 90% of CMP added was phosphorylated to CDP or CTP within 4 hr incubation in the reaction mixture at pH 5.8 or 5.9. This suggests that glucose fermentation and CMP phosphorylation were not affected even at the lower pH of the initial reaction mixture.

2) *Effect of phosphate buffer concentration:* Since the concentration of phosphate ions in the reaction mixture is one of the most important factors affecting the rate of glucose fermentation,<sup>28)</sup> the optimum concentration of phosphate ions for CDP-choline formation was investigated.

As shown in Fig. 3, the optimum concentration of phosphate ions was about  $3 \times 10^{-1}$  M. With increasing phosphate concentration, CMP phosphorylation was accelerated, while the production of CDP-choline was reduced. At lower phosphate concentration ( $10^{-1}$  M or  $2 \times 10^{-1}$  M), CDP-choline which accumulated in the early period of the reaction gradually decreased in the later stage of incubation and more than 50% of the CMP of the reaction mixture remained unphosphorylated, whereas high phosphate concentration ( $4 \times 10^{-1}$ ,  $5 \times 10^{-1}$ , and  $6 \times 10^{-1}$  M) caused a gradual increase of CDP-choline formation,

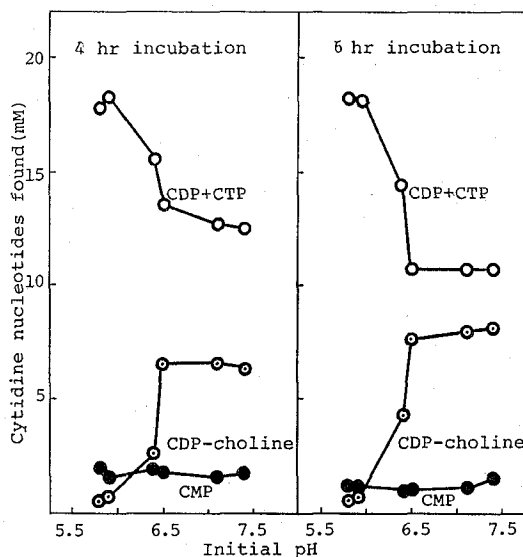


Fig. 2. Chemical changes in CDP-choline formation at different initial pH values.

Standard reaction mixture was used except that the pH of potassium phosphate buffer was varied.

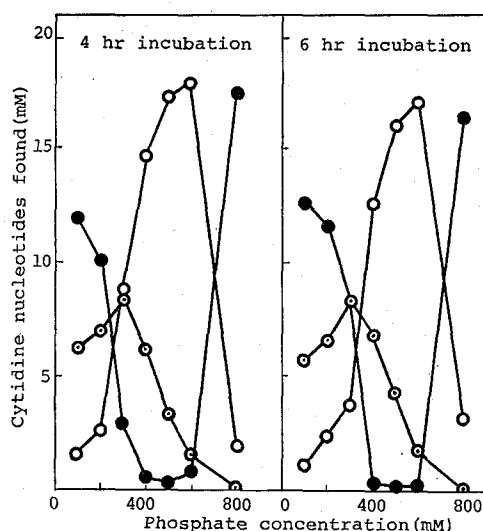


Fig. 3. Effect of phosphate concentration on chemical changes in CDP-choline formation.

The reaction mixture (mM): CMP, 20; choline, 50; glucose, 400;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 10; potassium phosphate buffer (pH 8.0) was varied as indicated.

Cells 100 mg per ml. Total volume 2.0 ml. Shaken at  $28^\circ\text{C}$  for 8 hr.

●—●, CMP; ○—○ CDP+CTP; ◐—◐, CDP-choline.

and CMP in the reaction mixture was almost completely phosphorylated to CDP and CTP within 4 hr incubation.

3) *Glucose concentration*: The effect of glucose concentration was investigated. The optimum concentration was more than  $6 \times 10^{-1}$  M. As shown in Fig. 4, at concentrations of less than  $4 \times 10^{-1}$  M, CDP-choline which formed in the early stage of the reaction gradually decreased on further incubation, but glucose concentrations of more than  $5 \times 10^{-1}$  M caused gradual increase of the production of CDP-choline which amounted to about 11 mM under these conditions.

4) *Magnesium concentration*: Magnesium ions are known to be an indispensable factor of choline phosphorylation,<sup>29-31)</sup> glycolysis<sup>28,32)</sup> and formation of sugar nucleotides.<sup>16,17)</sup> The amount of CDP-choline produced within 4 hr incubation was proportional to the concentration of  $\text{Mg}^{2+}$ ; at the concentration of more than 20 mM, CDP-choline formation was neither accelerated nor inhibited (Fig. 5).

5) *Choline concentration*: The effect of choline concentration on CDP-choline formation was investigated. As shown in Fig. 6, the optimum concentration of choline was  $8 \times 10^{-2}$  M, which corresponded to 4 times the amount of CMP added. By use of a lower concentration of choline than  $2 \times 10^{-2}$  M, the amount of CDP-choline formed was about 50% of that from  $8 \times 10^{-2}$  M of choline. When more than  $8 \times 10^{-2}$  M of choline was added, the amount of CDP-choline formed was not increased.

6) *CMP concentration*: The percentage of CMP converted into CDP-choline formed was compared. Table II shows that a lower concentration of CMP added was more effectively converted to CDP-choline. More than 80% conversion to CDP-choline was obtained from  $5 \times 10^{-3}$  M of CMP, but when  $5 \times 10^{-2}$  M of CMP was added less than 20% was converted. The maximum yield of CDP-choline of  $1.1\text{--}1.3 \times 10^{-2}$  M was obtained



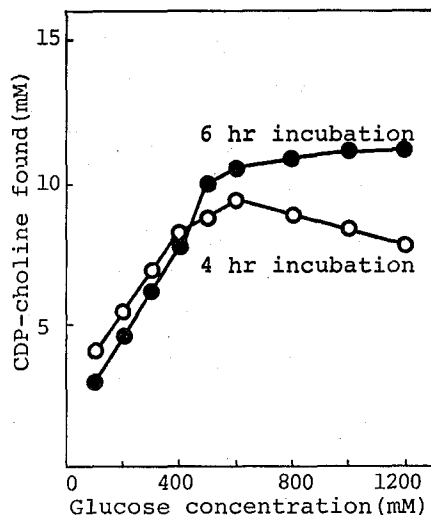


Fig. 4. Effect of glucose concentration on CDP-choline formation.  
The reaction mixture (mM): CMP, 20; potassium phosphate (pH 8.0), 300; choline, 50;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 10; glucose was varied as indicated above.  
Cells 100 mg per ml. Total volume 2.0 ml. Shaken at 28°C.

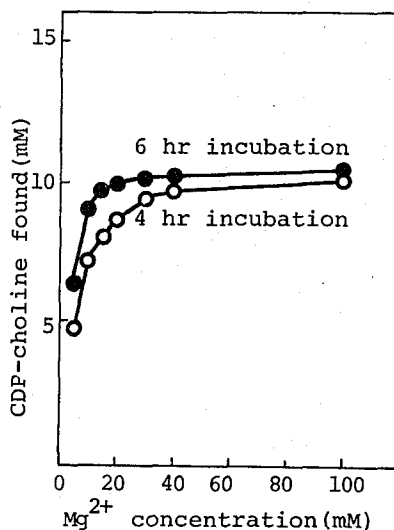


Fig. 5. Effect of magnesium concentration on CDP-choline formation.  
The reaction mixture (mM): CMP, 20; choline, 50; potassium phosphate (pH 8.0), 300;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , was varied as indicated above.  
Cells 100 mg per ml. Total volume 2.0 ml. Shaken at 28°C.

at  $2 \times 10^{-2}$  M of CMP added. Inhibition of CDP-choline formation by high concentration of CMP added will be discussed in the following paper.

From the data presented above, the optimum conditions for the CDP-choline formation by *Hansenula jadinii* IFO 0987 was summarized as shown in Table III.

7) *Cultivation time of cells*: The activity of CDP-choline formation sometimes varied with different dried cell preparations. It was found that the variation in the activity was

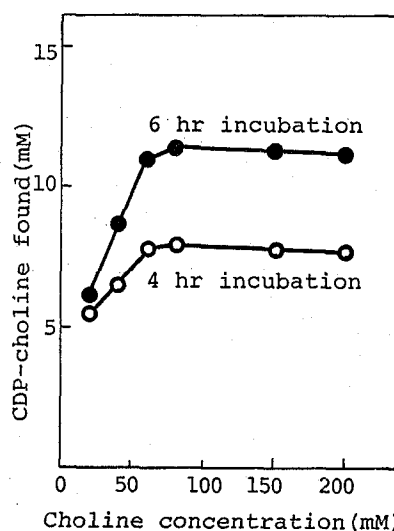


Fig. 6. Effect of choline concentration of CDP-choline formation.

The reaction mixture (mM): CMP, 20; glucose, 600; potassium phosphate (pH 8.0), 300;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 30; choline was varied as indicated above.

Cells 100 mg per ml. Total volume 2.0 ml. Shaken at 28°C.

Table II. The Percentage of CMP Converted into CDP-choline.

The reaction mixture contained (mM): glucose, 600; choline, 80;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 30; dried cells 100 mg per ml. Total volume 2.0 ml. Incubated at 28°C for 6 hr.

CMP added (mM)	CDP-choline found (mM)	Percentage converted (%)
5	4-4.5	80-90
12	6.5-7.5	55-65
20	11-13	55-65
30	10-12	33-40
40	9-10	22-25
50	8-9	15-18

Table III. Composition of Reaction Mixture Suitable for CDP-choline Formation.

5'-CMP	20 mM
Choline chloride	80
Glucose	600
Potassium phosphate buffer (pH 8.0)	300
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	30
Cells	100-200 mg/ml
Total	2.0 ml

caused by differences in cultivation time of the cells.

As shown in Fig. 7, activity of CDP-choline formation was decreased in both the choline system and the P-choline system in proportion to the increased cultivation time.

# CDP-Choline Production from CMP and Choline by Yeasts

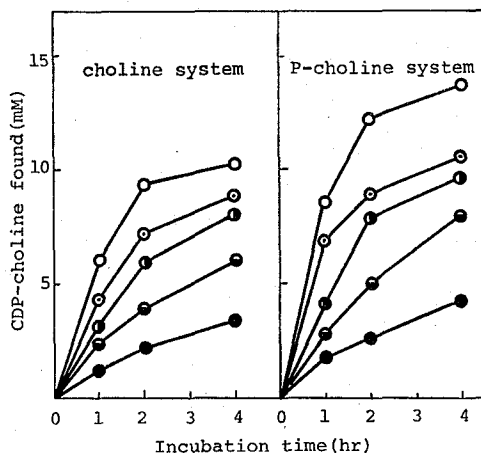


Fig. 7. Effect of cultivation time of cells on CDP-choline formation. The reaction mixture (mM): CTP, 20; glucose, 600; potassium phosphate (pH 8.0), 300;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 30; choline, 80 in choline system; P-choline, 50 in P-choline system. Cells 100mg per ml. Total volume 2.0ml. Shaken at 28°C. Cultivation time: ○—○, 18 hr; ⊙—⊙, 24 hr; ●—●, 36 hr; ●—●, 48 hr; ●—●, 72 hr.

Chemical change of CDP-choline formation in the choline system of 18 hr cultured cells was compared to that of 72 hr cultured cells. As shown in Fig. 8, the phosphorylation of CMP to CDP or CTP occurred to the same degree, but the formation of CDP-choline by 72 hr cultured cells was 50% of that by 18 hr cultured cells. The same results were obtained when choline was replaced with P-choline (Fig. 7). The rate of CDP-choline formation was decreased in inverse proportion to the cultivation time of the cells. The amount of CDP-choline formed by 72 hr cells at 4 hr incubation was less than 30% of that

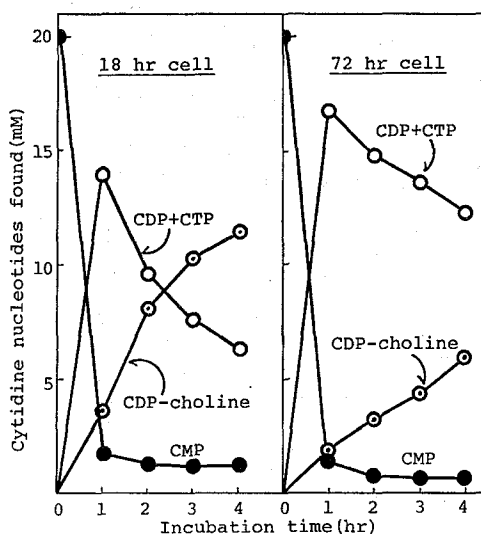


Fig. 8. Chemical changes in CDP-choline formation between 18 hr cultured cells and 72 hr cultured cells. The reaction mixture is same as that of Fig. 7.

of 18 hr cells. These facts suggest that while no differences occurred in the CMP phosphorylating system in those two cell preparations, longer cultivation time contributed significantly to the loss of both choline kinase and P-choline cytidyl transferase activity.

Choline kinase activity of cells cultured for different periods was measured by the phosphorylation of choline and ethanolamine by use of the modified reaction mixture without addition of CMP. Phosphorylcholine and phosphorylethanolamine produced are presented in Table IV. Activity of 24 hr cultured cells was 96% of that of 18 hr cultured cells, and 78%, 56%, and 44% of activity was observed with 36, 48, and 72 hr cultured cells, respectively. It is evident that both choline kinase and CDP-choline cytidyl transferase were inactivated by longer cultivation times.

8) *Effect of cell concentration*: The effect of cell concentration on CDP-choline formation was investigated by use of 18 hr cultured cells. Figure 9 (A-E) shows the relationship between cell concentration and CDP-choline formation. The rate of CMP phosphorylation and glucose catabolism, which is closely related to the energy supplying system, affected the rate of CDP-choline formation.

CDP-choline formation was linearly proportional to the incubation time on addition of more than 100 mg per ml of cells (Fig. 9-A). The amount of CDP-choline formed in the reaction mixture containing more than 100 mg per ml of dried cells reached 9 to  $12 \times 10^{-3}$  M on incubation for 2.5 hr, but when 50 mg per ml of cells was added, a sigmoidal curve of CDP-choline formation was observed and the yield of the product was only about  $3 \times 10^{-3}$  M. About 50% of CMP remained in the reaction mixture after 1 hr incubation when 50 mg per ml of cells was used (C), while more than 90% of CMP was phosphorylated in the reaction mixture of 100 mg cells per ml. Although more than  $16 \times 10^{-3}$  M of CDP+CTP were accumulated after 1.5 hr incubation with 50 mg per ml of cells, a considerable amount of CDP-choline was not formed. The low yield of CDP-choline with 50 mg per ml of dried cells suggests an insufficiency of the enzyme necessary to form CTP or P-choline.

Glucose consumption relates closely to the accumulation of FBP, which also relates to the supply of phosphorylation energy for both CMP and choline. Glucose consumption of 50 mg per ml of cells at 2.5 hr was less than 50% of that of 100 mg per ml of cells, and FBP accumulated at 1 hr incubation was less than 15% of that of 100 mg per ml of cells.

Under the conditions presented here, the optimum cell concentration for CDP-choline formation was decided at more than 100 mg per ml.

9) *Isolation and identification of CDP-choline produced*: For the isolation and identi-

Table IV. Effect of Cultivation Time on the Choline kinase Activity.

Cultivation time (hr)	18	24	36	48	72
Compound	Phosphorylated compound (mM)				
Choline	27	26	21	15	12
Ethanolamine	6	3	3	3	2

Reaction mixture contained (mM): glucose, 600; potassium phosphate (pH 8.0), 300;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 30; choline or ethanolamine, 100; cells 100 mg/ml. Total 2.0 ml. Reaction was carried out for 6 hr with shaking at 28°C.

# CDP-Choline Production from CMP and Choline by Yeasts

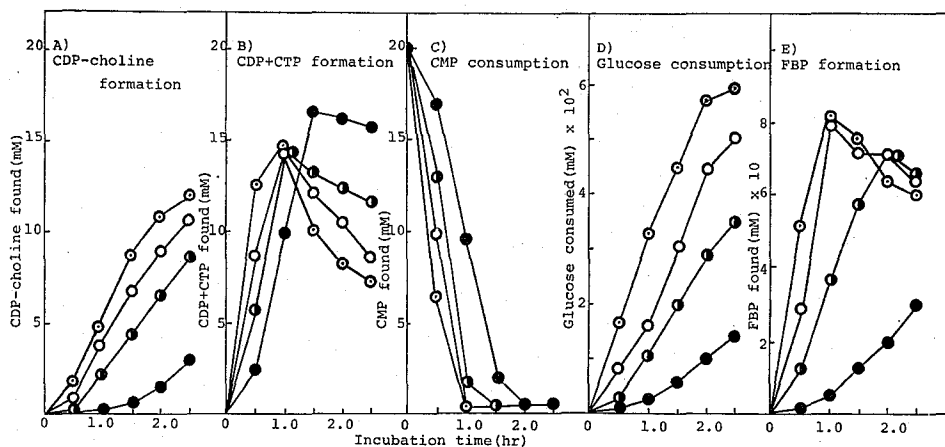


Fig. 9. Effect of cell concentration on chemical changes in CDP-choline formation. Composition of the reaction mixture was the same as shown in Fig. 7. The cell concentration (18 hr cultured) was varied as indicated below:  
 ●—●, 50 mg per ml; ◐—◐, 100 mg per ml; ○—○, 150 mg per ml; ◑—◑, 200 mg per ml.

fication of CDP-choline, a large scale reaction was performed. The composition of reaction mixture employed was as given in Table II. Incubation was maintained for 6 hr at 28°C with shaking, and 2 m moles of CMP were used.

After the treatment of the centrifuged supernatant of the reaction mixture by the procedure stated in the method, CDP-choline was isolated as its sodium salt giving 120 mg of white, plate-like, somewhat hygroscopic crystals.

The crystals melted at 266–267°C and decomposed at 269°C. The isolated nucleotide had  $E_{\max}$  at 280 nm and  $E_{\min}$  at 241 nm in  $10^{-2}$  N HCl, the absorption spectrum being identical with that of cytidine (Fig. 10). The elution pattern of the nucleotide is shown in Fig. 11.

Infra-red spectrum of the product is presented in Fig. 12. The typical peak at  $1660\text{ cm}^{-1}$  represents the  $\text{O}=\text{C}-\text{N}$  group of the pyrimidine ring, and the peaks at 1220–

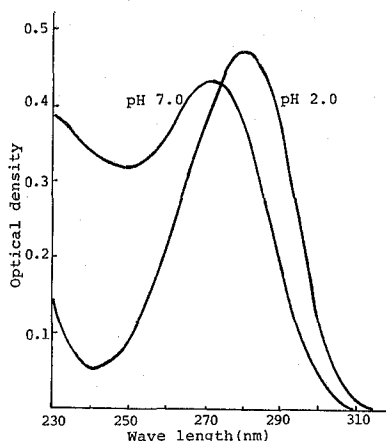


Fig. 10. Ultra violet absorption spectra of the product under acidic and neutral conditions.

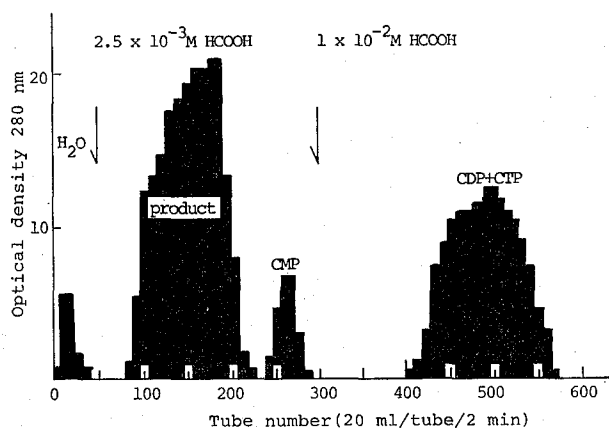


Fig. 11. Elution pattern of cytidine nucleotides from Dowex-1, X-2 (formate form).

1240, 1080 and  $920-940\text{ cm}^{-1}$  represent  $\text{N}^+(\text{CH}_3)_3$ ,  $\text{O}-\text{P}(=\text{O})(\text{OH})-\text{O}-\text{CH}_2-$ , and  $\text{O}-\text{P}(=\text{O})(\text{OH})-\text{O}-\text{P}(=\text{O})(\text{OH})-$ , respectively.

Proton magnetic resonance spectrum of the product is presented in Fig. 13. The signal at  $\delta=3.19$  of 9 protons was assigned to the  $\text{N}^+(\text{CH}_3)_3$  group, the doublet signal of  $\delta=6.05$  and  $1.14$  was assigned to the 5 proton of the pyrimidine ring, and the doublet signal at  $\delta=7.9$  and  $8.0$  was assigned to 6 proton of the pyrimidine ring, and signal at  $\delta=5.98$  was assigned to the 1'-ribose. The other signals were assigned to other protons as indicated in the Figure. These data support the fact that the product is composed of P-choline and CMP.

Paper chromatographic analysis of the isolate and its acid-hydrolysate are shown in Table V. The  $R_f$  value of the isolate was almost identical with that of authentic CDP-choline, and CMP was liberated from the isolate when it was hydrolyzed in 1 N HCl at  $100^\circ\text{C}$  for 10 min. One mole of CMP was released from one mole of the isolate by the hydrolysis, and had an  $R_f$  value identical with that of authentic P-choline (Table VI). The amount of P-choline released was determined by the above mentioned method.

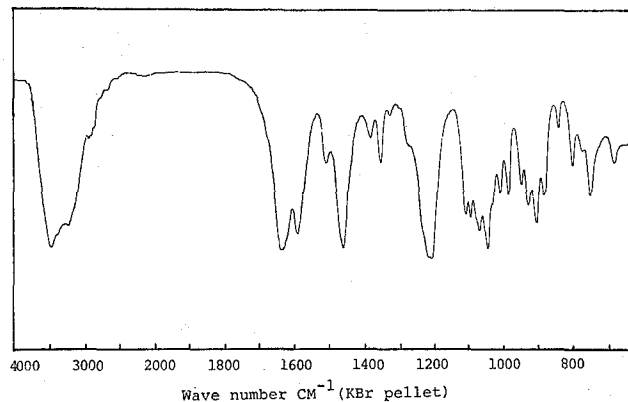


Fig. 12. Infra-red absorption spectrum of CDP-choline Na-salt.

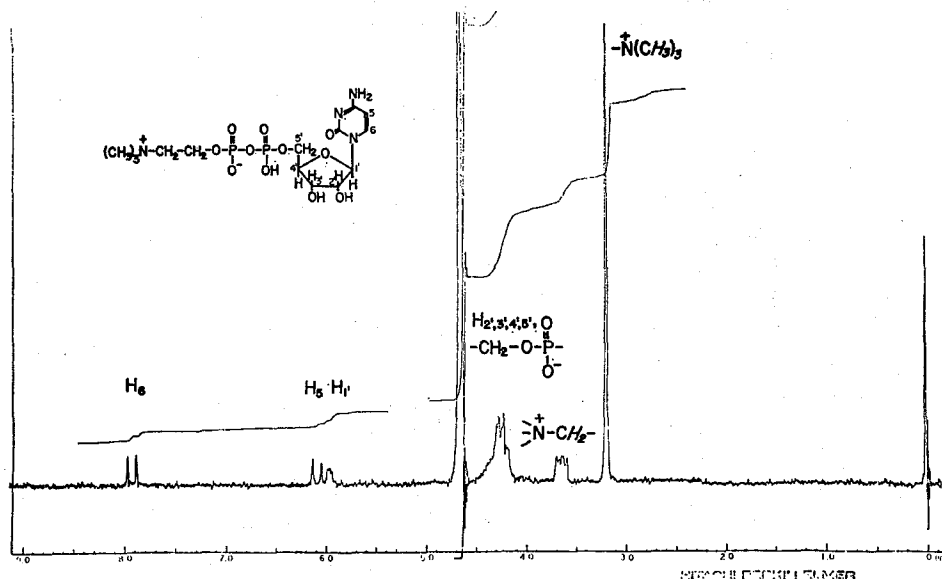
PMR spectrum of CDP-choline ( $D_2O$ , 90MHz)

Fig. 13. PMR spectrum of CDP-choline Na salt.

About one mole of P-choline was liberated from one mole of the isolate calculated as cytidine nucleotide from its aqueous solution.

Elementary analysis showed C=26.22%, H=5.47%, and N=9.43%. A molecular formula of  $C_{14}H_{28}N_4P_2O_{11}Na_2 \cdot 6H_2O$  was calculated. The number of moles of water of crystallization of the CDP-choline salt was calculated from the UV absorptivity of the acidic solution of the product. From the results described so far, it was concluded that the isolated nucleotide is CDP-choline disodium salt.

Table V. Paper Chromatographic Identification of the Product and Its Hydrolysate\*

Compounds	R <sub>f</sub> values		
	I**	II***	III****
PRODUCT	0.44	0.41	0.36
Hydrolysate*	0.27	0.39	0.08
5'-UMP	0.27	0.29	—
5'-CMP	0.27	0.39	0.08
CDP-choline	0.44	0.41	0.36
CDP-ethanolamine	0.40	0.41	0.39

\* The product was hydrolysed with 1 N HCl at 100°C for 120 min.

\*\* 95% ethanol: 1 M ammonium acetate (pH 7.0), (2 : 1).

\*\*\* isobutyric acid and 0.5 N  $NH_4OH$  (10 : 6).

\*\*\*\* isopropanol: conc.  $NH_4OH$ :  $H_2O$  (7 : 1 : 2).

Table VI. Paper Chromatographic and Thin Layer Chromatographic Identification of Aminoethanol Moiety of the Product.

	R <sub>f</sub> values		
	PPC* (a)	(b)	TLC**
Choline	0.60		0.51
Dimethylethanolamine	0.50		0.59
monomethylethanolamine	0.30		0.70
Ethanolamine	0.11		0.79
P-choline		0.50	
P-ethanolamine		0.32	
Hydrolysate***		0.50	
Enzyme treated hydrolysate****	0.60		0.51

\* (a) phenol (50 g): *n*-butanol (50 ml): 80% formic acid (3 ml): H<sub>2</sub>O (10 ml).

(b) 95% ethanol: 1 M ammonium acetate (pH 7.0), (2 : 1).

\*\* Methanol: conc. HCl (95 : 5).

\*\*\* The product was hydrolyzed with 1 N HCl at 100°C for 120 min.

\*\*\*\* The acid hydrolysate was treated with *E. coli* phosphatase after neutralization.

## DISCUSSION

Fermentative production of CDP-choline from choline and CMP by choline kinase-linked system was investigated using dried cell preparations of yeasts. It was found that *H. jadinii*, *C. utilis*, *S. carlsbergensis*, *S. cerevisiae* and *S. rouxii* were capable of CDP-choline formation from choline and CMP. Only one strain, one of the strains of *C. utilis*, showed higher activity of choline kinase than *H. jadinii*.

Results suggested that choline kinase activity is distributed widely among yeasts, but is not strong. It is of interest that although *T. candida* produced CDP-choline in quantity from P-choline and CMP,<sup>11)</sup> little production was observed from choline and CMP, which implies that *T. candida* has no capacity for choline phosphorylation.

The rate of CDP-choline formation from choline and CMP was sensitive to the lowering of the pH of reaction mixture. The fact that CDP-choline formation is sensitive to acidic conditions corresponds well to the sensitivity of choline kinase to acidic conditions.<sup>29,30)</sup>

Phosphate ion concentration is one of the most important factors affecting the fermentative production of CDP-choline. At the concentration of less than  $3 \times 10^{-1}$  M, CDP-choline formation proceeded immediately by phosphorylation of CMP, but in the presence of more than  $4 \times 10^{-1}$  M of phosphate ions, the rate of CDP-choline formation decreased, even though large amounts of CDP and CTP were formed.

*H. jadinii* could not catabolyze glucose in phosphate buffer of concentration higher than  $6 \times 10^{-1}$  M. With phosphate buffer of  $8 \times 10^{-1}$  M, no FBP accumulation and no phosphorylation of CMP were observed. The ability of *H. jadinii* to catabolyze glucose was less than that of baker's yeast which accumulates FBP under high phosphate conditions.<sup>28)</sup>

Although high concentration of CMP was phosphorylated to CDP or CTP, the conversion ratio of CMP to CDP-choline decreased with higher concentrations of CMP.



The maximum transformation of CMP to CDP-choline was about 65% at the concentration of  $2 \times 10^{-2}$  M. Inhibition of choline kinase by CTP will be discussed in the following paper.

Since the activity of both choline kinase and CDP-choline-cytidyl transferase of *H. jadinii* depended on the cultivation time, it is necessary to avoid long time cultivation for the preparation of dried cells. Glucose catabolism and CMP phosphorylation were not, however, affected by long time cultivation.

### SUMMARY

Fermentative production of CDP-choline by yeast from choline and CMP by a choline kinase-linked system was investigated. Yeasts having marked activity of CDP-choline formation and choline phosphorylation were sought.

*H. jadinii* was selected as a suitable strain of CDP-choline forming yeast. Other than *H. jadinii*, *C. utilis* IFO 0396, IFO 0639, IFO 1086, *H. beijerinckii* IFO 0981, *H. miso* IFO 0146, *S. carlsbergensis* IFO 0641, *S. cerevisiae* IFO 0021, *S. rouxii* IFO 0320 and IAM 4369 showed comparable activity of CDP-choline formation.

CDP-choline production by *H. jadinii* was strictly controlled by pH of the reaction mixture, CMP concentration, phosphate ion concentration and cultivation time of the cells. When the pH of the initial reaction system was lower than 6.5, CDP-choline formation was remarkably reduced. Long time cultivation of cells resulted in significant reduction in the activity of both choline kinase and CDP-choline-cytidyl transferase. The phosphorylation of CMP was not affected by long time cultivation.

Under higher concentration of phosphate ions than  $4 \times 10^{-1}$  M, the rate of glucose dissolution gradually fell and caused decrease of CMP phosphorylation.

The optimum conditions for CDP-choline formation were as follows: CMP, 20 mM; choline, 80 mM; potassium phosphate (pH 8.0), 300 mM; glucose, 600 mM;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  30 mM; and dried cells 100–200 mg per ml in a total volume of 2.0 ml.

More than 13 mM of CDP-choline was produced under the optimum condition.

The method presented in this paper will be developed to provide easy production of  $^{32}\text{P}$ -labeled or  $^{14}\text{C}$ -labeled CDP-choline and other CDP-aminoethanols.

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